EFFECTS OF INTRAUTERINE GROWTH RESTRICTION AND A MATERNAL HIGH FAT DIET ON EPIGENETIC DETERMINANTS IN RAT LUNG

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Intrauterine growth restriction (IUGR) complicates up to 15% of pregnancies and often occurs in conjunction with exposure to a maternal diet high in saturated fats (HFD). IUGR is associated with alterations to the structure and development of the lung. Lung development depends upon the precise and coordinate expression of genetic and epigenetic determinants. Methylation of lysine 20 on histone 4 (H4K20Me) is an epigenetic histone modification of interest in lung development. The enzyme Setd8 places the H4K20Me mark along target genes, including the PPARγ gene. The H4K20Me mark increases transcription of PPARγ mRNA and production of the translated product, PPARγ protein. By way of a feedback loop, PPARγ is also the transcription factor that regulates transcription of Setd8. PPARγ is critical in alveolarization, or the final stage in lung development. We previously showed that a diet high in *unsaturated* fat can reverse effects of IUGR on lung molecular changes, however, the effects of a diet high in *saturated* fats is unknown. In this study we hypothesized that maternal HFD in combination with IUGR will decrease PPARγ2 and Setd8 mRNA and protein expression beyond that of IUGR alone. We hypothesize that H4K20Me in whole rat lung and at Exon 4 of the PPARγ gene will also be decreased by HFD and IUGR. Sprague-Dawley rats were studied before and after alveolarization (prealveolarization and postalveolarization). Dams either underwent uteroplacental insufficiency to induce IUGR or anesthesia only

for control rats. Half of each group (IUGR and control) were fed a regular diet while the other half were fed a HFD from preconception to the end of the study. Our study demonstrated that, HFD in combination with IUGR (HFD-IUGR) affects the PPARγ-Setd8 feedback loop in rat lung in a sex-specific manner. In male rat lung, HFD-IUGR resulted in disconnects in the PPARγ-Setd8 feedback loop. Furthermore, disconnects in the PPARγ-Setd8 feedback loop persisted beyond alveolarization. These novel findings suggest that male rat lungs are more susceptible to diet induced changes in the PPARγ-Setd8 feedback loop than female rat lungs. Further, these changes may make male IUGR rat lungs susceptible to lung injury over the longterm.

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INTRODUCTION

Intrauterine Growth Restriction and Lung Development

Up to 15% of pregnancies in the United States are complicated by intrauterine growth restricted (IUGR). IUGR is characterized by the failure of a fetus to reach its full genetic growth potential [1]. IUGR results in increased complications early on and into adult life [1-3]. Maternal malnutrition and/or reduced placental transport of nutrients are often underlying factors contributing to IUGR [2]. In the United States and other countries of affluence, IUGR is typically the result of poor maternal-fetal transfer as a consequence of maternal hypertension. IUGR as a result of maternal hypertension typically results in asymmetric growth restriction, with normal head size and reduced stature and weight [3].

In early life, premature newborns often suffer respiratory insufficiency requiring ventilator support. Approximately 5-15% of premature newborns are also IUGR [1]. IUGR infants that are born premature have an increased risk of chronic lung disease that persists into adulthood [3]. Bronchopulmonary dysplasia (BPD) is the most common complication of birth in preterm infants [4]. The National Institute of Child Health and Human Development diagnoses BPD if the infant remains oxygen dependent at 28 days of life [5-7]. Alternatively BPD can be diagnosed based on the criterion of oxygen dependency at 36 weeks

postconceptional age [4, 7]. Low gestational age is a major predictor of BPD [4, 8, 9], additionally **IUGR has been identified as an independent risk factor for development of BPD** [8, 10]. Many infants with BPD remain oxygen dependent for several months and are readmitted to the hospital within the first few years of life [6]. Children who were diagnosed with BPD in infancy, have lower lung volumes, decreased gas mixing efficiency and suffer more coughing/wheezing [6]. Lung impairment in BPD infants persists through adulthood [2, 4-6].

Lung impairment in BPD infants is associated with alterations to the structure of the lung. In human lung, the saccular stage of lung development occurs in weeks 24-36 of gestation. Saccular development is characterized by thinning of epithelial cells, terminal saccular formation, and surfactant production[11]. The alveoli, gas exchanging units of the lung, are formed *in utero* and continuing developing new alveoli until 18-36 months of life in human infants [2, 11] . Lung growth after this point is mainly through enlargement of existing alveoli [2]. Lung development in rats occurs in stages similar to human development, as outlined in Figure 1. However in rats, saccular development continues through about postnatal day 5 and alveolar development occurs from about postnatal day 5 through day 30.

In newborn rat pups, IUGR results in altered lung structure. Our lab has previously found that IUGR increases distal airspace wall thickness in the lung of newborn rat pups [12, 13]. IUGR decreases postnatal lung development in female pups[13]. In newborn female rat lung, the saccular walls are thicker in IUGR female pups compared to female controls. No alterations in lung structure

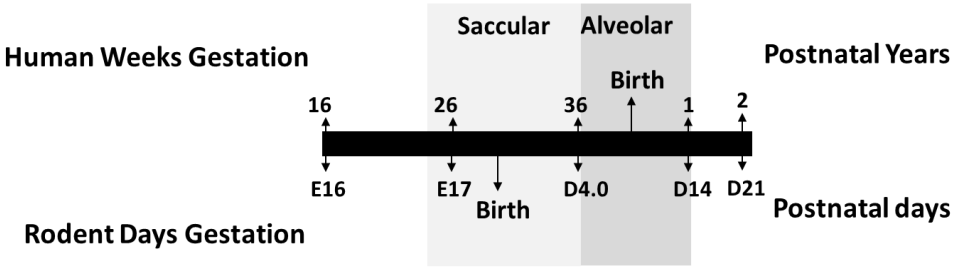

Figure 1. Timeline of Lung Development

were observed in male pups. Alterations in lung structure likely contribute to the comorbidities associated with IUGR.

The development of appropriate lung structure depends upon the precise regulation of expression of genes coordinating lung development. Regulation of gene expression is governed by epigenetic modifications.

Epigenetics

Epigenetic modifications are heritable alterations that effect gene expression but leave the DNA sequence unchanged [14]. In eukaryotic cells, DNA is packaged tightly in units known as chromatin, as shown in Figure 2. Chromatin consists of a series of subunits known as nucleosomes. Each nucleosome contains two copies of each of the four histone proteins: H2A, H2B, H3, and H4. DNA is tightly coiled around the histone proteins [15]. Methylation of histone proteins is one epigenetic modification that can occur in response to environmental changes.

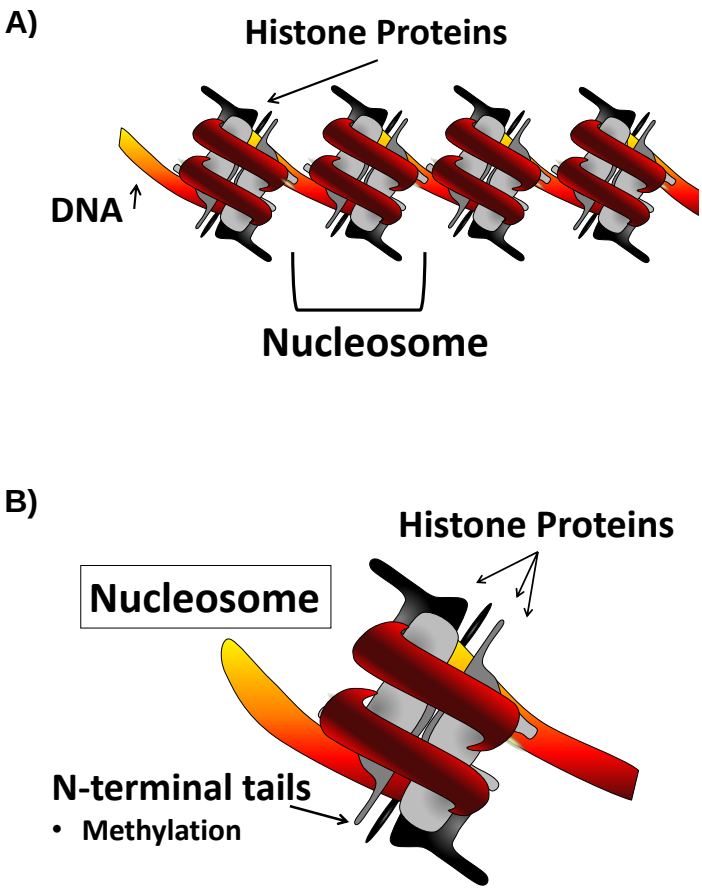

Figure 2. Schematic of DNA Packaging.
A) Chromatin structure. B) Closer look at the nucleosome subunits.

Methylation of lysine 20 on histone 4 (H4K20Me) is a histone modification of interest in lung development. H4K20Me is placed by the histone lysine methyltransferase Setd8. Setd8 is the only enzyme known to place the H4K20Me mark [16]. An important target of Setd8 is the peroxisome proliferator-activated receptor gamma (PPARγ) gene. Setd8 places the H4K20Me mark along the PPARγ gene, resulting in increased transcription of PPARγ mRNA and production the translated product, PPARγ protein [16]. At the same time, PPARγ is also a transcription factor that regulates the transcription of Setd8. Thus,

PPARγ and Setd8 act together in a feedback loop, see Figure 3 [16]. Of relevance to this study, PPARγ is well known for its role in lung development [17, 18]

PPARγ and the Lung

The ligand activated transcription factor, PPARγ has been identified as critical in pre- and postnatal lung development [17-21]. PPARγ isoforms are expressed in pulmonary inflammatory, mesenchymal and epithelial cells [18]. This study will focus on the PPARγ2 isoform. PPARγ knockout mice present with immature enlarged airspaces resulting in increased lung volume and decreased tissue resistance [18]. Additionally, PPARγ is a necessary component in the parathyroid hormone related protein-driven epithelial-mesenchymal paracrine loop that contributes to lung development [22].

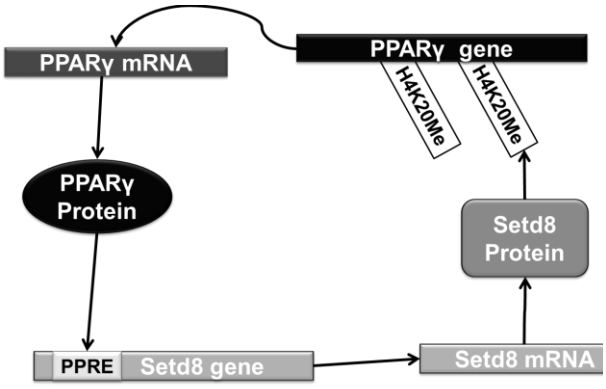

Figure 3. PPARγ-Setd8 Feedback Loop

In order for PPARγ to function in lung development, agonists must be available to activate PPARγ. PPARγ agonists include the polyunsaturated fatty acid docosahexaenoic acid (DHA). Notably, DHA is reduced in serum of pregnant women with preeclampsia [23]. During the last trimester of pregnancy, maternal-placental transfer of DHA is increased but maternal plasma DHA is reduced in mothers of IUGR and small for gestational age newborns [17].

Preliminary Data

We previously demonstrated, in a rat model of IUGR, that IUGR decreases H4K20Me in newborn male rat lung relative to male control rat lungs. In addition, IUGR decreased H4K20Me in both male and female rat lung at Exon 4 of the PPARγ gene. This decrease was found in conjunction with a decrease in levels of the PPARγ target gene, Setd8. In the same rat model of IUGR, rat pups are deficient in DHA at birth, and maternal DHA supplementation was found to restore DHA serum levels in the newborn infant, as well as restore H4K20Me on the PPARγ gene, as well as levels of Setd8 and PPARγ to that of a control animal [17].

High Fat Diet and Lung Development

While a diet high in *unsaturated* fats, specifically DHA, can ameliorate the effects of IUGR on H4K20Me, Setd8, and PPARγ, the typical Western diet is high in *saturated* fats (HFD). The effect of high fat diet throughout life increases risk of chronic disease and is generally associated with a lower quality of life.

It is well-known that rates of obesity and overweight are on the rise in the United States and around the world. Among women of childbearing age, 24.5% are overweight and 23.0% are obese according to the 2002 National Survey of Family Growth [24]. This increase in obesity rates is often associated with the increased prevalence of a HFD diet. At the same time, rates of preeclampsia and gestational hypertension in the United States are increasing [25]. As previously discussed, these maternal vascular diseases are a common cause of IUGR. While the effects of obesity and HFD in general are associated with short- and longterm health effects for mother and child, it is **unknown how HFD alone or in combination with IUGR will affect PPARγ, Setd8 and H4K20Me.**

Hypothesis

We hypothesize that, in rat lung, maternal HFD in combination with IUGR will decrease PPARγ2 and Setd8 mRNA and protein expression beyond that of IUGR alone, both before and after alveolarization. We hypothesize that H4K20Me in whole rat lung and at Exon 4 of the PPARγ gene will also be decreased by HFD and IUGR at the same time points.

METHODS

Model

IUGR was induced by uteroplacental insufficiency (UPI) in Sprague-Dawley rats as previously described by our group [17, 19, 26-28]. In brief, on day 19 of gestation (E19) dams were anesthetized with ketamine and xylanine and underwent bilateral uterine artery ligation. Control dams underwent the same anesthetized procedures at E19. Rat pups were delivered by c-section on E21.5, or day 0 (D0) of life. Rat pups were humanely killed and whole lungs removed and flash-frozen in liquid nitrogen and stored at -80C. Day 21 (D21) rat pups were allowed to be delivered naturally at term and were nursed by their dams until time of tissue harvest at D21. All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the American Physiological Society's guiding principles.

Diet

Dams were fed either Regular diet (RD) or HFD prior to mating and during gestation through D21, weaning, Figure 4. RD rat chow consists of 29% kcals protein, 54% kcals carbohydrate, 17% kcals fat (primarily soybean oil and 0.04% wt/wt cholesterol), resulting in a caloric density of 3 kcal/g. HFD is 16% kcals protein, 30% kcals carbohydrate, 44% kcals fat (primarily milk fat, 65% saturated

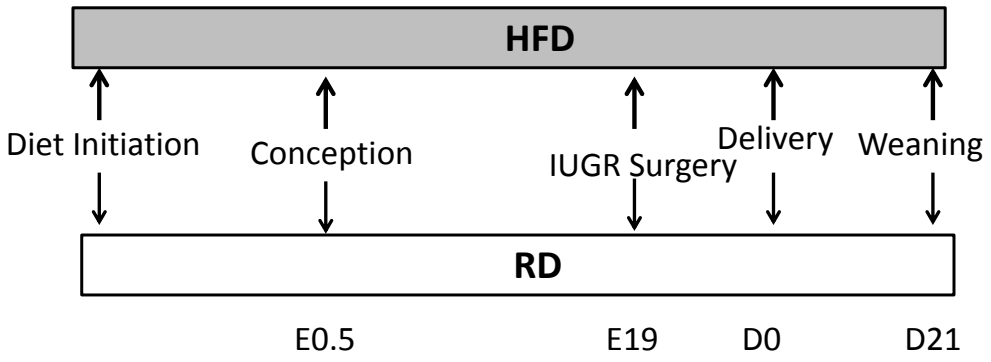

Figure 4. Intervention Initiation Timeline

fat, 1% wt/wt cholesterol, and 0.5% wt/wt cholic acid) resulting in a caloric density of 4.3 kcal/g. The two diets were fed to both UPI and control dams to create four groups as described in Table 1.

Real-time RT PCR

PPARγ2 and Setd8 mRNA levels were evaluated via real-time RT- PCR in whole lung of D0 and D21 rats from each of the four groups, separated by sex. The housekeeping gene, GAPDH, was used as an internal control. The following primer/probe sets were used: PPARγ2–Rn00440940_m1, Setd8–Rn01477383_g1(Applied Biosystems, Carlsbad, CA). GAPDH primer and probe sequences; Forward:CAAGATGGTGAAGGTCGGTGT; Reverse: CAAGAGAAGGCAGCCCTGGT; Probe: GCGTCCGATACGGCCAAATCCG. Total RNA was extracted from frozen Con, IUGR, HFD-Con, and HFD-IUGR lung using the RNAeasy Mini Kit (Qiagen, DB Biosciences, CA) according to manufacturer's instruction. Total RNA was quantified using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized

Table 1
Study Groups

Group	Surgery	Diet
Con	Anesthesia only	Regular diet prior to conception through weaning
IUGR	Bilateral uterine artery ligation	Regular diet prior to conception through weaning
HFD-Con	Anesthesia only	High fat diet prior to conception through weaning
HFD-IUGR	Bilateral uterine artery ligation	High fat diet prior to conception through weaning

using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster CA) from 1µg of total RNA.

All real-time PCR amplification, data acquisition and analysis were done using the 7900HT Real-time PCR system and SDS Enterprise Software (Applied Biosystems, Carlsbad, CA) using a 384-Well Optical Reaction Plate (Applied Biosystems, Carlsbad, CA). Taqman Universal PCR Mastermix (Applied Biosystems, Carlsbad, CA) was used in a 6µL reaction, performed in quadruplicate. Cycle parameters were: 50°C × 2 min, 95°C × 10 min, followed by 40 cycles of 95°C × 15 sec and 60°C × 60 sec.

Western Blotting or Immunoblotting

Immunoblotting was used to determine levels of whole lung PPARγ2 and Setd8 protein in in Con, IUGR, HFD-Con, and HFD-IUGR rats. Total protein was isolated from whole lung tissue by homogenizing approximately 25mg tissue in

RIPA buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 0.5% Na-deoxy-cholate, 1% NP-40 (Igepal), 0.1% SDS) and protease inhibitor cocktail (PIC) (Roche-Complete Mini, Roche Applied Science, Indianapolis, IN); followed by centrifugation at 10,000g, 4°C, 15 minutes. Supernatants were collected and stored at −80°C until use. Protein was assayed in triplicate for protein concentration using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL).Total protein (25 µg) was loaded and separated by Nu-Page 10% Bis-Tris Midi Gel (Novex by Life Technologies, Grand Island, NY) at 150 V for 60 minutes. After gel electrophoresis, proteins were transferred to PVDF membranes (Milli-pore, Billerica, MA) at 4°C for 1 hour at 100 V. Membranes were then blocked in 5% milk TBS-T for 1 hour then washed multiple times in 1X TBS-T. After blocking, bound proteins were exposed to antibodies against GAPDH (Abcam, Cambridge, MA) 1:5,000 in 5% milk with PPARγ 1:500 in 5% milk or with Setd8 1:8000 in 5% milk. Blots were incubated overnight at 4°C. After multiple wash steps in TBS-T, membranes were probed with appropriate secondary antibody: antirabbit IgG antibody (Cell Signaling Technology, Danvers, MA) or bovine antigoat (Santa Cruz Biotechnology, Dallas, Texas) for 1 hour at room temperature. After multiple wash steps in TBS-T, antibody signals were detected with Western Lighting enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA) and quantified using an Image Station 2000R (Eastman Kodak/SIS, Rochester, NY). GAPDH signal was used to normalize samples.

Chromatin Immunoprecipitation

A revised ChIP protocol, based on the methods of Farnham and Bomsztyk [29], was used to investigate the levels of histone modifications along the PPARγ gene at the following positions: 5' region of promoter 1 (5′ of P1), promoter 1 (P1), promoter 2 (P2), Exon 4 and at the 3′ untranslated region (3' end), depicted in Figure 5. The five positions were chosen because they each represent areas of the PAPRγ gene subject to different transcriptional regulatory mechanisms.

Briefly, chromatin isolation from male and female whole lung (190–280 mg) was performed as follows; tissue was fixed in 1% formaldehyde for 10 minutes at room temperature, the reaction was stopped by the addition of glycine to a final concentration of 125 mM. Samples were centrifuged, washed twice with phosphate buffered saline (PBS) and resuspended in PBS with added PIC at manufacture's recommended concentration (Roche-Complete Mini, Roche Applied Science, Indianapolis, IN). After centrifugation, cell pellets were resuspended in lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Igepal) with

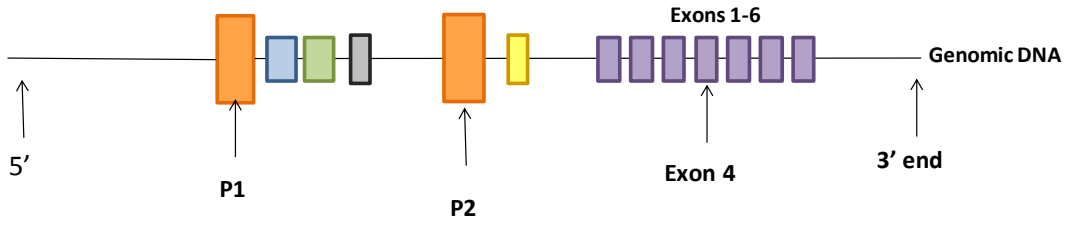

Figure 5. PPARγ Gene

PIC and dounced on ice using a tight pestle. After centrifuging, pelleted nuclei were resuspended in nuclei lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS) with PIC, incubated for 20 minutes on ice and split into aliquots of approximately 200 µl (in 1.5 ml tubes) for sonication. Sonication was performed on ice using a Fisher Scientific Model 100 sonicator with microtip attachment (Fisher Scientific, Pittsburgh, PA). Each sample was pulsed 10 times with the micotip placed near the base of the tube and returned to ice, this was repeated a total of eight times. After centrifuging, chromatin containing supernatants for each of the four samples were frozen at −80°C.

Immunoprecipitation (IP) were performed as previously [29] using anti-H4K20Me (NB 21-2088), A nontranscribing intergenic region was used as a control. Primers sequences used for RT-PCR of IP DNA are listed in Table 2.

Statistical Analysis

Test groups were compared to the corresponding sexmatched Con. Statview software was used for ANOVA analysis. A p-value of <0.05 was considered significant.

Table 2
Primer/probe Sets for ChIP RT-PCR

Transcript position relative to PPARγ gene	Sequence
5′ of P1	For: 5′ TCGACGGCTTTCTGAATGTG Rev: 5′ CTTGCCCTCTTTCAGCTCTTTC Probe: 5′ATCTTTAGGACAGATCATG
P1	For: 5′ AAAAACAAACTTCTGCGTGACAGT Rev: 5′ GGTCCCACGTTCCTCAGACA Probe: 5′ AGGGCACCAGCCGG
P2	For: 5′ CCAAGTCTTGCCAAAGAAGCA Rev: 5′ GATTGAGAGCCAGCTGTGACAA Probe: 5′ ACAGCATTATGACACACCAT
Exon 4	For: 5′ CCATCAGGTTTGGGCGAAT Rev: 5′ GATCTCCGCCAACAGCTTCT Probe: 5′ CCACAGGCCGAGAAG
3′ End	For: 5′ CGCCAAGGTGCTCCAGAA Rev: 5′ CTGCACGTGCTCTGTGACAA Probe: 5′ ATGACAGACCTCAGGCAG

RESULTS

Results will be presented for D0 (prealveolarization) data, followed by D21 (postalveolarization) data.

D0, Prealveolarization

D0 PPARγ2 mRNA and Protein

In male D0 rat lung, HFD-Con ($p=0.039$) and HFD-IUGR ($p=0.022$) decreased PPARγ2 mRNA relative to Con (Figure 6A). However, in male D0 rat lung PPARγ2 protein levels were unaffected by either IUGR or HFD-Con (Figure 6B). In female D0 rat lung, IUGR ($p<0.0001$), HFD-Con ($p=0.0002$), and HFD-IUGR ($p=0.0003$) decreased PPARγ2 mRNA levels relative to control (Figure 6C). In female D0 rat lung, IUGR ($p=0.0128$) and HFD-Con ($p=0.0053$) decreased PPARγ2 protein levels, relative to control (Figure 6D). The combination of HFD-Con and IUGR did not significantly alter PPARγ2 protein levels in female rat lung.

D0 Setd8 mRNA and Protein

In male D0 rat lung, HFD-Con ($p=0.0281$) and HFD-IUGR ($p=0.0040$) increased Setd8 mRNA relative to Con (Figure 7A). Similarly, HFD-Con

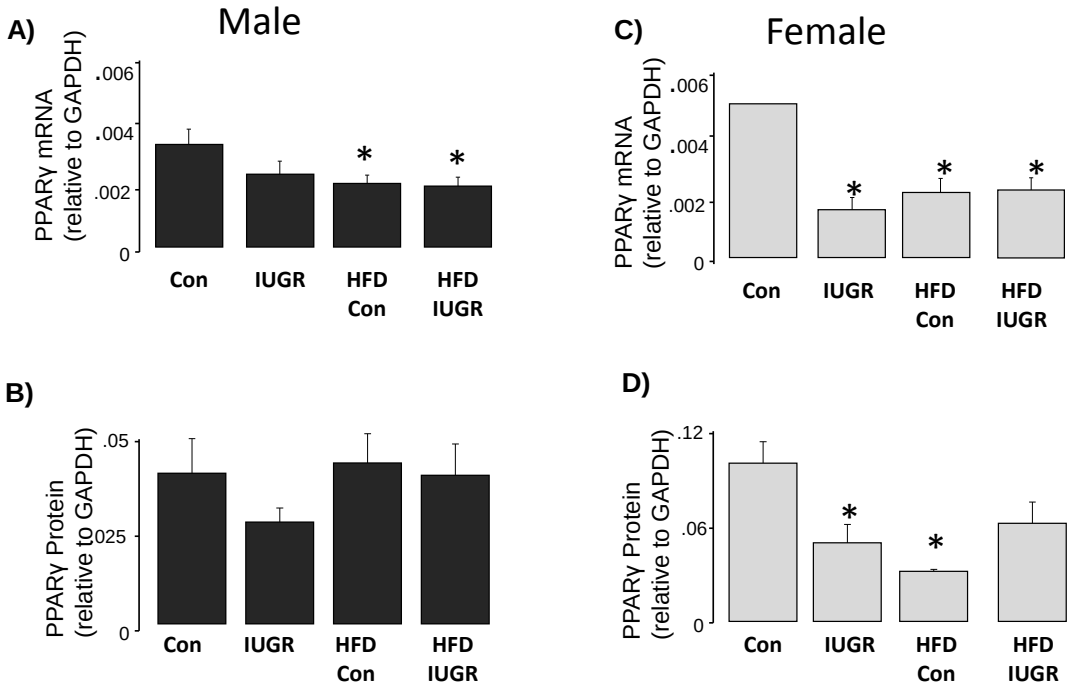

Figure 6. D0 PPARγ mRNA and Protein.
A) Male D0 PPARγ2 mRNA levels relative to GAPDH. B)Male D0 PPARγ2 protein levels relative to GAPDH. C) Female D0 PPARγ2 mRNA levels relative to GAPDH. D) Female D0 PPARγ2 protein levels relative to GAPDH. *Denotes statistical significance relative to sex-matched Con, $p<0.05$

(p=0.0014) and HFD-IUGR (p<0.0001) increased Setd8 protein relative to Con. HFD-IUGR (p=0.0181) increased Setd8 protein relative to HFD-Con (Figure 7B).

In female D0 rat lung, IUGR (p=0.0068) and HFD-IUGR (p=0.0133) decreased Setd8 mRNA relative to Con (Figure 7C). HFD-IUGR (p=0.0347) decreased Setd8 mRNA relative to HFD-Con. In female D0 rat lung, IUGR (p=0.0009) and HFD-Con (p=0.0190) decreased Setd8 protein relative to Con (Figure 7D).

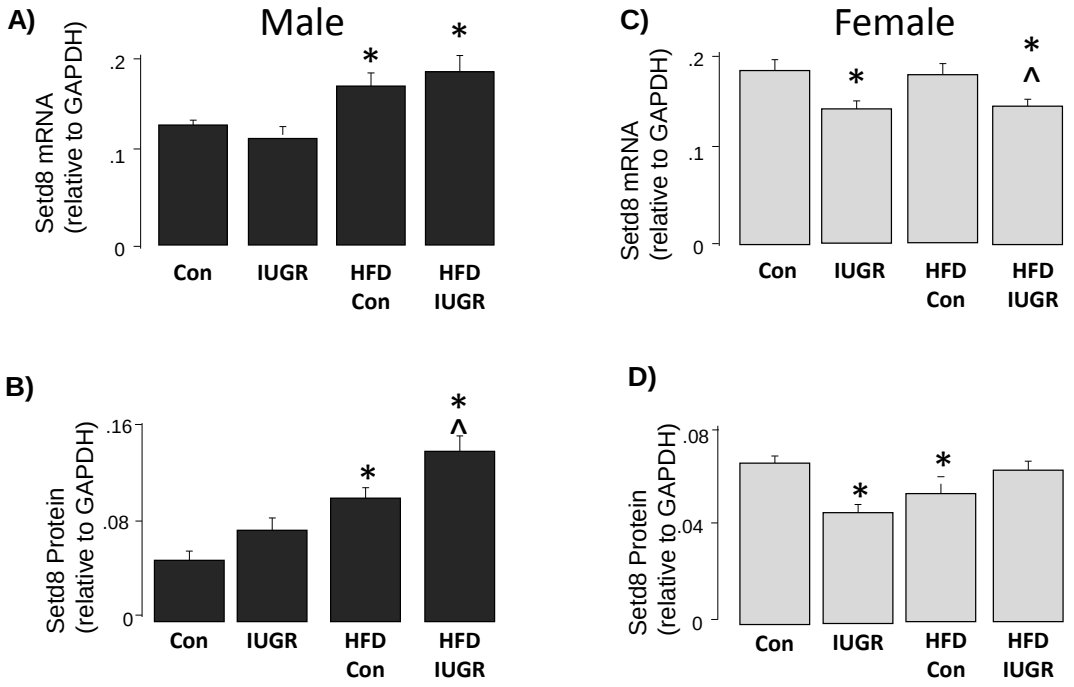

Figure 7. D0 Setd8 mRNA and Protein
A) Male D0 Setd8 mRNA levels relative to GAPDH. B) Male D0 Setd8 protein levels relative to GAPDH. C) Female D0 Setd8 mRNA levels relative to GAPDH. D) Female D0 Setd8 protein levels relative to GAPDH. *Denotes statistical significance relative to sex-matched Con, $p<0.05$. ^Denotes statistical significance relative to HFD-Con, $p<0.05$

<u>D0 H4K20Me</u>

H4K20Me was altered along the PPARγ gene, Figure 8. In male D0 rat lung, HFD-Con ($p=0.0088$) and HFD-IUGR ($p=0.0454$) both decreased H4K20Me in the 5' region of Promoter 1 and, Promoter 1 ($p<0.0001$) and in Promoter 2 ($p<0.001$). HFD-IUGR ($p=0.0317$) reduced H4K20Me in the 3' untranslated region. No changes where observed Exon 4 of the PPARγ gene in D0 male rat lung.

In female D0 rat lung, HFD-Con ($p=0.0086$) decreased H4K20Me in the 5' promoter region. HFD-Con and HFD-IUGR decreased H4K20Me in Promoter 1

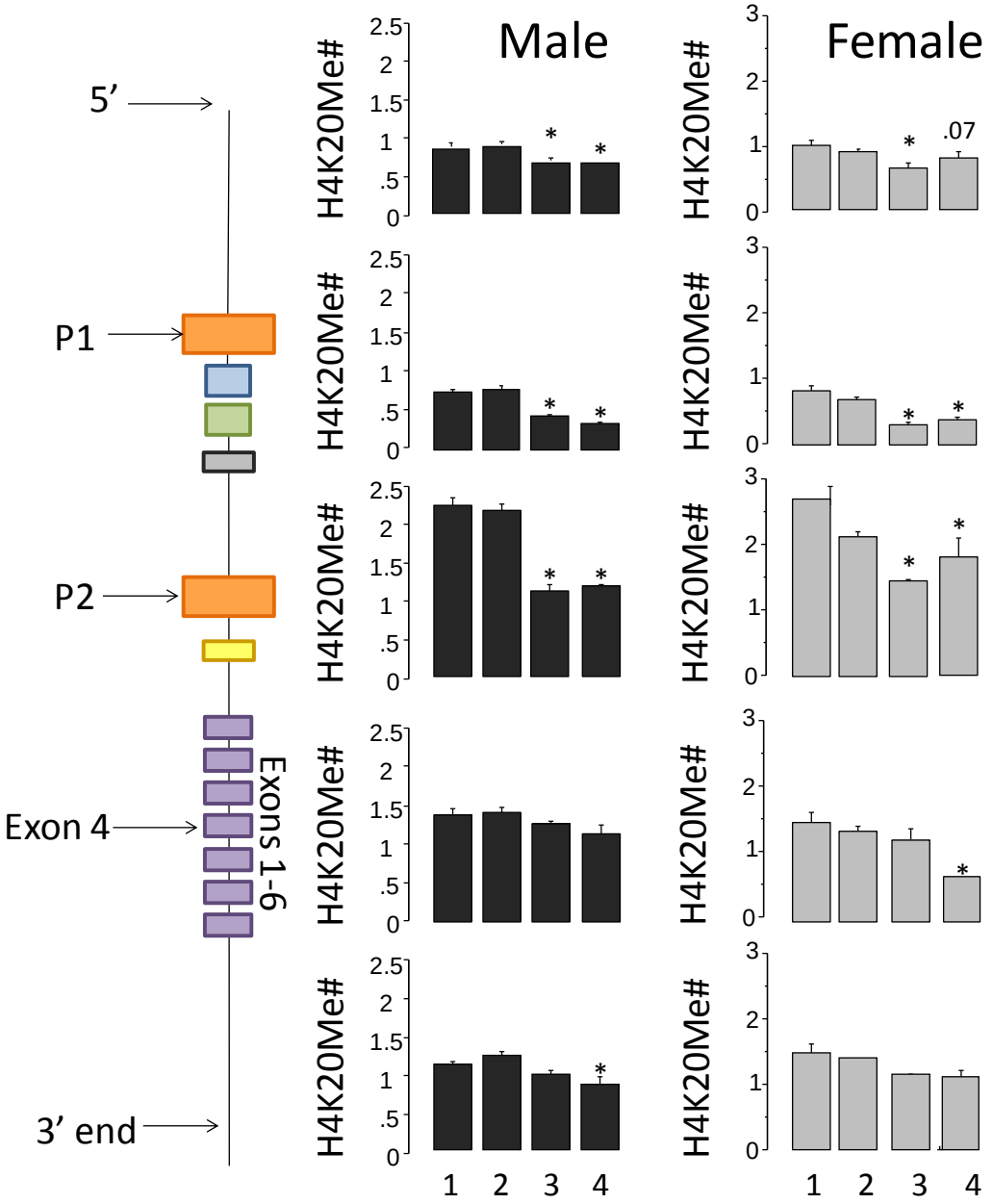

Figure 8. D0 H4K20Me Along PPARγ
Table Legend: 1=Con, 2=IUGR, 3=HFD-Con, 4=HFD-IUGR
relative to nontranscribing intergenic region
*Denotes statistical significance relative to sex-matched Con, p<0.05

(p<0.0001 and p<0.0001, respectively) and in Promoter 2 (p=0.0285 and H4K20Me in Exon 4 (p=0.0261)). The 3' untranslated region was unaffected by the test groups.

D21, Postalveolarization

D21 PPARγ2 mRNA and Protein

In male D21 rat lung, IUGR (p=0.0035) and HFD-Con (p=0.0010) decreased PPARγ2 mRNA relative to Con (Figure 9A). HFD-IUGR (p=0.0940) did not alter PPARγ2 mRNA relative to Con. However, in male D21 rat lung PPARγ2 protein levels were not affected by IUGR, but HFD-Con (p=0.0198) and HFD-IUGR (p=0.0016) decreased PPARγ2 protein relative to Con (Figure 9B). In female D21 rat lung, only HFD-IUGR (p=0.0364) decreased PPARγ2 mRNA levels relative to Con (Figure 9C). In female D21 rat lung, IUGR (p<0.0001) increased PPARγ2 protein levels, relative to Con (Figure 9D).

D21 Setd8 mRNA and Protein

In male D21 rat lung, Setd8 mRNA was unchanged in all groups relative to Con (Figure 10A). IUGR (p=0.0130) and HFD-IUGR (p=0.0001) increased Setd8 protein relative to Con. HFD-IUGR (p=0.0032) increased Setd8 protein relative to HFD-Con (Figure 10B). In female D21 rat lung, Setd8 mRNA was unchanged in all gropus relative to Con (Figure 10C). Accordingly in female D21 rat lung, Setd8 protein was unchanged in all groups relative to Con (Figure 10D).

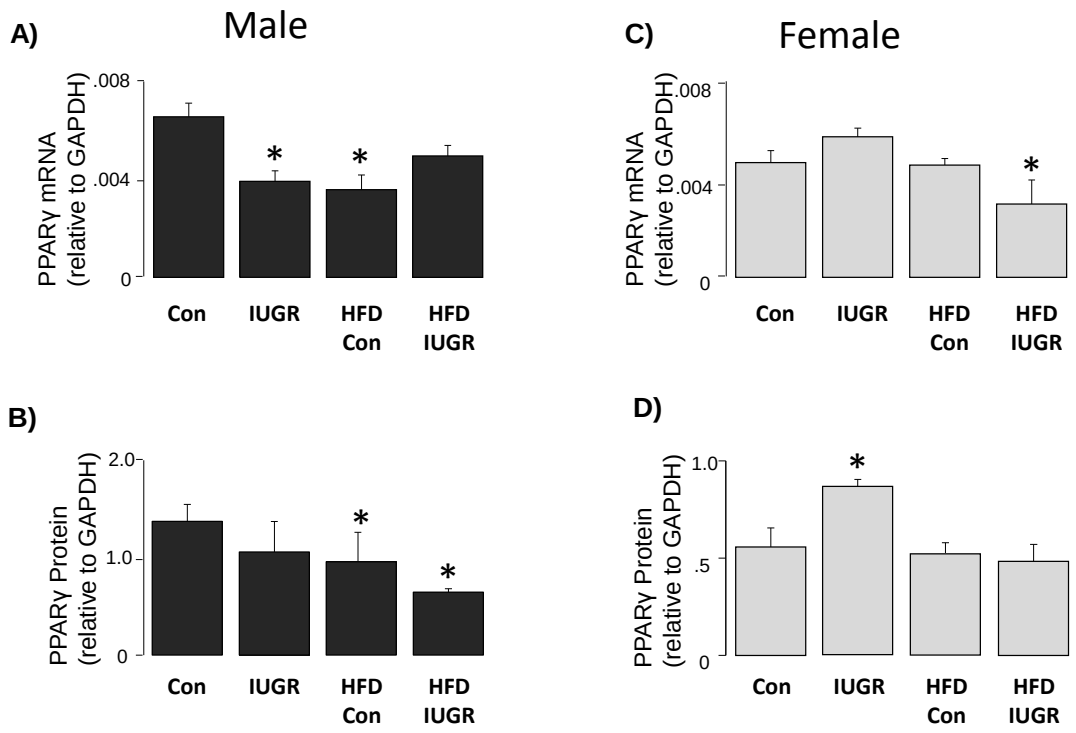

Figure 9. D21 PPARγ mRNA and Protein
A) Male D21 PPARγ mRNA levels relative to GAPDH. B) Male D21 PPARγ protein levels relative to GAPDH. C) Female D21 PPARγ mRNA levels relative to GAPDH. D) Female D21 PPARγ protein levels relative to GAPDH. *Denotes statistical significance, p<.05

D21 H4K20Me

H4K20Me was altered along the PPARγ gene, Figure 11. In male D21 rat lung, HFD-Con and HFD-IUGR decreased H4K20Me in the 5' promoter region (p=0.0042 and p=0.0010 respectively), in Promoter 1 (p=0.0049 and p=0.0002, respectively), and in Promoter 2 (p=0.0379 and p=0.0008, respectively). In male D21 rat lung HFD-IUGR decreased H4K20Me compared to Con (p=0.0024) and HFD-Con (p=0.0008) at Exon 4. In male D21 rat lung, HFD-IUGR decreased

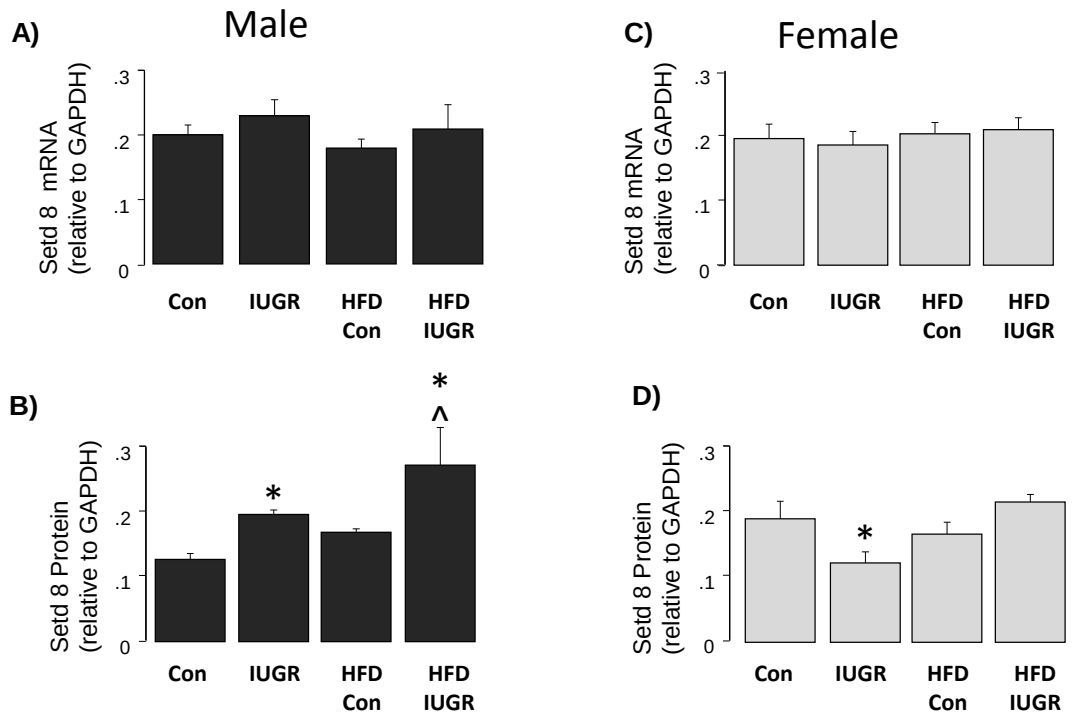

Figure 10. D21 Setd8 mRNA and Protein
A) Male D21 Setd8 mRNA levels relative to GAPDH. B) Male D21 setd8 protein levels relative to GAPDH. C) Female D21 Setd8 mRNA levels relative to GAPDH. D) Female D21 Setd8 protein levels relative to GAPDH. *Denotes statistical significance, p<.05. ^Denotes statistical significance relative to HFD-Con, p<.05.

H4K20Me in the 3' untranslated region compared to Con (p=0.0336) and HFD-IUGR (p=0.0289).

In female D21 rat lung, neither IUGR or HFD, alone or in combination, changed H4K20Me in the 5' promoter region, Promoter 1, Promoter 2, Exon 4 or the 3' untranslated region compared to Con. In female D21 rat lung, HFD-IUGR (p=0.0437) decreased H4K20Me in Promoter 1 compared to HFD-Con. In female D21 rat lung, HFD-IUGR (p=0.0440) decreased H4K20Me in the 3' untranslated region compared to HFD-Con.

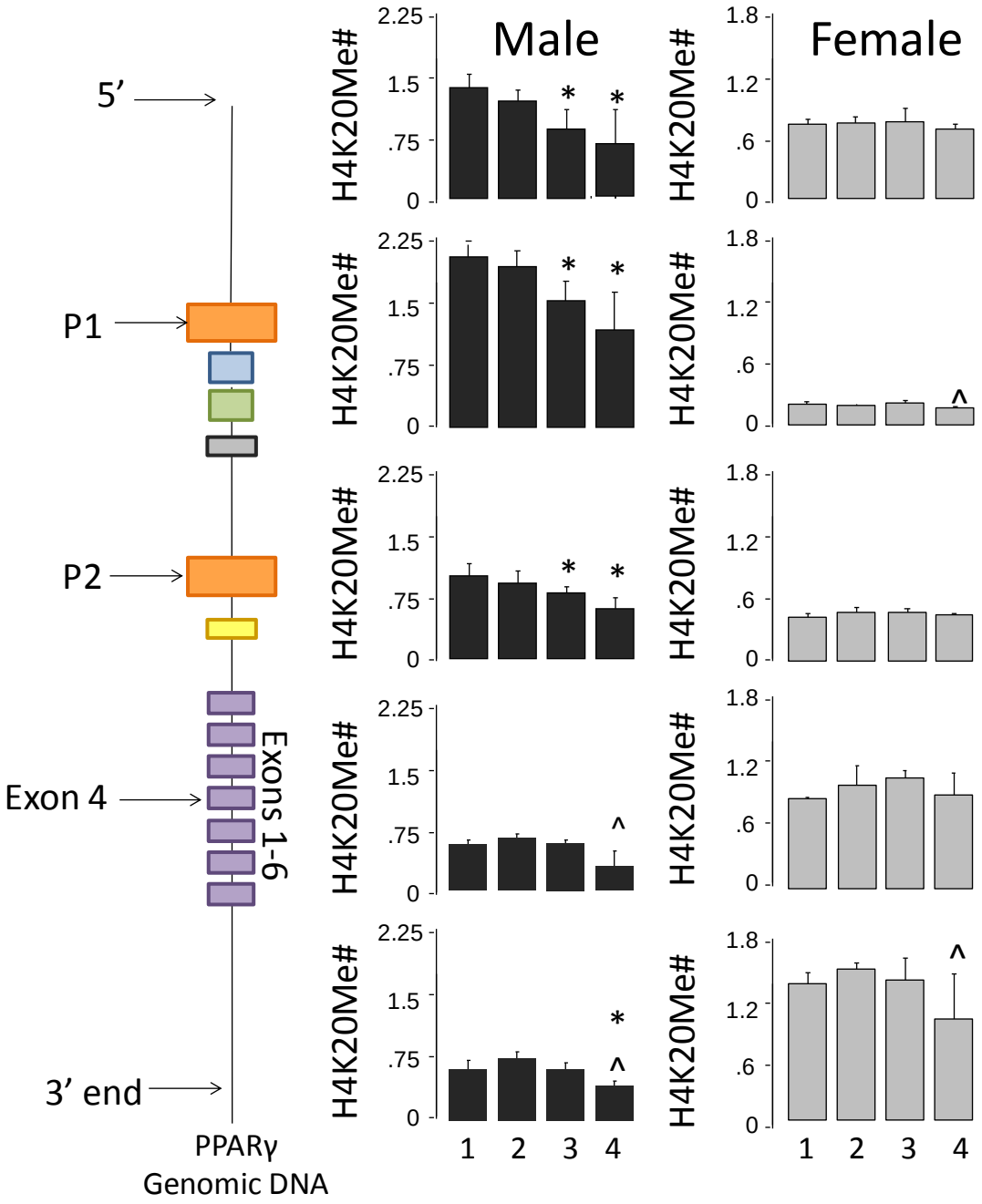

Figure 11. D21 H4K20Me Along PPARγ
Table Legend: 1=Con, 2=IUGR, 3=HFD-Con, 4=HFD-IUGR
relative to nontranscribing intergenic region
*Denotes statistical significance relative to sex-matched Con, p<0.05
^Denotes statistical significance relative to sex-matched HFD-Con, p<0.05

DISCUSSION

Our study revealed important and novel findings. HFD in combination with IUGR (HFD-IUGR) alters the PPARγ-Setd8 feedback loop in rat lung in a sex-specific manner. In male rat lung, HFD-IUGR disrupts interactions between components of the PPARγ-Setd8 feedback loop. Importantly, the effects of HFD-IUGR on the PPARγ-Setd8 feedback loop in male rat lung persist beyond alveolarization. These novel findings suggest that male rat lungs are more susceptible to diet induced changes in the PPARγ-Setd8 feedback loop than female rat lungs. Further, these changes may make male IUGR rat lungs susceptible to lung injury over the longterm.

HFD-IUGR affects the PPARγ-Setd8 feedback loop differently in male and female rat lung. In female rat lung, HFD-IUGR reduced PPARγ2 and Setd8 mRNA expression, and reduced H4K20Me on the PPARγ gene. These results suggest that in female HFD-IUGR rat lung, the PPARγ-Setd8 feedback, while reduced in magnitude compared to female control lung, maintains appropriate interactions between components of the feedback loop. These HFD-IUGR data, in female rat lung, are consistent with our previous study of IUGR alone. This suggests that in female rat lung, HFD does not alter the response to IUGR. Furthermore, significant effects of IUGR and HFD-IUGR, in female rat lung, do not extend beyond the newborn period.

However, in male rat lung, HFD with or without IUGR, resulted in an unanticipated disconnect in the PPARγ-Setd8 feedback loop. HFD-IUGR decreased PPARγ2 expression as expected in newborn male rat lung. However this was associated with twofold *increase* in Setd8 mRNA and protein compared to sex matched controls. Despite the increased levels of Setd8 protein, HFD-IUGR reduced H4K20Me along the PPARγ gene in newborn male rat lung. This suggests that in males a HFD interferes with the interactions between PPARγ2 expression, Setd8 expression and placement of the H4K20Me mark on the PPARγ gene. One question raised by the observation of high Setd8 levels with low H4K20Me is why is not Setd8 methylation of the PPARγ gene reflective of these increased levels? While the present study was not designed to investigate this mechanism, potential factors can be hypothesized.

A decrease in H4K20Me under conditions of increased Setd8, could be the result of several situations. First, Setd8 may not be able to place the H4K20Me mark because another chromatin modifying enzyme may have placed a different mark on H4K20 preventing access to Setd8. Additionally, HFD-induced alterations elsewhere in the histone code may impact the chromatin structure and prevent Setd8 from accessing the H4K20. Expression of other chromatin modifying enzymes known to impact Setd8 may be effected by a HFD. For example, overexpression of the di-/tri- methyltrnasferase, SUV4-20H, results in decreased levels of H4K20Me [30]. Alternatively, Setd8 may require assistance from other transcription factors or chromatin modifying enzymes that are not available due to changes triggered by a HFD. Yet, another alternative is

that Setd8 is still placing the mark effectively but its turnover is increased. The histone demethylase PHT8 can remove the H4K20Me placed by Setd8 [30], and it is unknown how HFD effects this histone demethylase.

In this study, we examined the effects on H4K20Me on the PPARγ gene, however, we did not examine how other target genes of Setd8 are affected. Setd8 function is indicated in the regulation of wnt signaling and in the transcriptional regulation of genes important in cellular differentiation. Wnt signaling and cellular differentiation are important to proper lung development [31].

A novel finding of this study is that the molecular changes elicited by HFD-IUGR persist postalveolarization in male rat lung. At D21HFD-IUGR rat lung is characterized by reduced PPARγ protein and increased Setd8 protein. Again, despite increased Setd8 protein, HFD-IUGR reduces H4K20Me along the PPARγ gene. The effects of decreased H4K20Me along the PPARγ gene on lung structure and function are currently unknown. However, we speculate that a persistent change in the level of expression of PPARγ and in the epigenetic H4K20Me profile of the PPARγ gene, will result in persistent changes in lung structure and function. These changes in lung structure and function may make the male rat lung more susceptible to lasting effects of injury. In fact, our lab has demonstrated that IUGR induced decreased PPARγ and H4K20me is associated with an exacerbated response to postnatal lung injury, in male rat lung but not female rat lung [32].

Sex differences are not always investigated in early developmental studies because these processes are often viewed as common to both male and female fetuses. However, recent research in our lab has shown that IUGR sex specifically alters expression of aromatase, an estrogen synthase, in newborn rat lung in association with sex specific changes in local estrogen:testosterone ratios [33]. Local changes in estrogen:testosterone ratios may be the initiator of sex specific differences in gene expression in the rat lung. Further studies are needed to investigate the presence of estrogen and testosterone response elements on genes implicated in maintenance of the PPARγ-setd8 feedback loop.

Strengths and Limitations

Our study is not without limitations. Firstly, we did not characterized lung structure and function in our rat model. Our molecular observations show that expression of genes important for lung development are dysregulated, implying that lung structure, and thus function, may also be impaired by HFD. It will be important for future studies to examine lung structure and function in HFD and HFD-IUGR rats. Caution should be taken when extrapolating data obtained in the rat, to human findings. However, lung development in both humans and rats follow the same pathway, differing only in timing relative to birth. One advantage of this difference is that the newborn rat lung is developmentally equivalent to a human lung at gestational week 28-32, a time when many IUGR infants are also

born preterm. This developmental timing allows us to evaluate the immature lung in the newborn rat, without the complications of prematurity.

Our lab has previously shown that IUGR, without alterations in diet, decreases PPARγ mRNA and protein levels in both male and female newborn rat lung [17]. Our current study is consistent with the previous work for female rat lung. However, in the current study, newborn male rat lung changes in PPARγ and Setd8, while in a similar direction, were not statistically significant. Failure to obtain statistical significance in male rat lung PPARγ and Setd8 decreases in the current study is likely a result of insufficient power resulting from too few samples. Repeating the experiment with a larger sample size will be important.

Potential Impact

Alterations in lung development resulting from prenatal insult increase the risk of chronic comorbidities and early mortality. Many women of childbearing age are at an increased risk of developing preeclampsia during pregnancy due to prepregnancy overweight and obesity. As a result, their unborn children are at an increased risk of being IUGR. Additionally, many women of child bearing age in the United States enjoy the typical Western diet which is commonly described as a HFD, placing their children at risk for exposure to a HFD *in utero*. **Because of the high potential of prenatal exposure to IUGR with a HFD, it is important to understand how these two unique prenatal insults act together.** Understanding how HFD and IUGR cause complications and alter lung development can help us understand how to potentially reverse or prevent these

deleterious effects. Current research is investigating potential markers for intrauterine growth restriction which may allow for interventions *in utero*, preventing the deleterious effects of IUGR and/or HFD [34, 35]. Increased understanding for the role of diet in lung development may also influence recommendations for ‘catchup’ growth in early life when lung development is still occurring and a high calorie HFD is often prescribed.

In conclusion, our study provides novel evidence that male and female rat lung responded differently to a HFD and HFD-IUGR. This suggests that male and female lungs are programmed to respond differently to injury and would likely respond differently to treatment. As we continue to extend upon the understanding of prenatal development it is necessary to evaluate differences between the sexes. Clinicians should be aware that sex may impact response to maternal diet as well as potential treatment options.

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